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Antimicrobial Effectiveness of Lysozyme Immobilized on Polyvinylalcohol-Based Film against *Alicyclobacillus acidoterrestris*

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ABSTRACT

In this study, the effectiveness of an active polyvinylalcohol-based film against *Alicyclobacillus acidoterrestris* was assessed. The active film was fabricated by immobilizing an active compound on the surface of a polymeric matrix and then tested by putting the film in contact with a medium that had been inoculated with microbial cells. Microbiological tests showed that the film was antimicrobial against both a single strain and a culture cocktail of *A. acidoterrestris*, at 44°C. By monitoring the viable cell concentration under three different packaging conditions, it was possible to demonstrate that the active film was equally effective against both the single strain and the culture cocktail and that it maintained this efficacy at various medium volumes. The same microbial tests were also conducted on viable spores of the investigated microorganism, inoculated both into a laboratory medium and apple juice. The results indicate that these viable spores were better inhibited than cells by the active film in both investigated media.

One of the emerging areas for research in the fields of both applied microbiology and packaging is the development of antimicrobial films for food packaging applications. In an active system, the package, the product, and the environment interact to prolong shelf life or to enhance the safety or sensory properties of the product while maintaining its quality *(1, 12).* In particular, antimicrobial packaging is designed to extend the lag phase and reduce the growth rate of microorganisms as an alternative to direct additives for minimizing microbial load *(5, 10, 13, 14).* Organic and inorganic compounds, acids, bacteriocins, enzymes, and naturally derived extracts from plants are some of the numerous active agents that can be used in the development of a film with antimicrobial properties *(20).*

Presently, two types of antimicrobial food packaging exist: (i) those that contain active agents that migrate, in a controlled or uncontrolled manner, to the surface of the food, and (ii) those that are effective against the surface growth of microorganisms without being released. Several studies have been reported in the literature that deal with release systems, because this is the more convenient means by which antimicrobial activity can be achieved *(6–9, 15, 16, 21).*

In Europe, these release systems have seen relatively little commercial success because of the lack of legislation; however, a technique that is based on the immobilization of the antimicrobial agent is desirable. A valid method for attaching a bacteriocin to a plastic film has been described by An et al. *(2),* who coated low-density polyethylene films with a mixture of polyamide resin and bacteriocin solution and then studied the antimicrobial activity of this agent against *Micrococcus flavus.* Low-density polyethylene films have also been successfully coated with nisin, in which derived cellulose materials were the carrier *(10).* An immobilization that is based simply on the adsorption of the active agent into the polymeric matrix has been tested on cellulose-based paper *(18).* Concerning surface immobilization, one of the most recent studies reported in the literature deals with the immobilization of antimicrobial peptides onto a polystyrene substrate *(4).* The results obtained show that part of the active substance is released into the aqueous solution in which the active film is immersed. Another aspect that must be considered is the potential for a reduction in antimicrobial activity due to immobilization of the active agent on the matrix. For some enzymes, changes in conformation and denaturization by solvents may result in low activity per unit area *(22).*

One of the most promising natural antibacterial agents is lysozyme, a single-peptide protein, which possesses enzymatic activity against the β-1–4 glycosidic linkages between the *N*-acetylmuramic acid and *N*-acetylglucosamine groups found in the peptidoglycan of bacteria. Lysozyme is a compound that has been widely researched in the development of potential antimicrobial food packaging materials; however, to our knowledge, very little studies have been made with respect to its immobilization on polymer surfaces *(3).*

In this study, the effectiveness of an active film based on the immobilization of lysozyme onto a polyvinylalcohol-based polymeric surface was tested against a spore-forming bacterium, *Alicyclobacillus acidoterrestris,* which is rec-

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ognized as a cause of spoilage in acidic beverages. Because of its thermal and acidity resistance, the selected microorganism is considered a target when quality control in acidic juices is important *(17)*. The effectiveness of the developed active film in slowing the growth of *A. acidoterrestris*–viable cells and spores was assessed in both a simulated food system and a commercial apple juice.

MATERIALS AND METHODS

Film preparation. The films studied were fabricated with polyvinylalcohol (molecular weight, 70,000 to 100,000 Da; Sigma-Aldrich, Gallarate, Italy) as the polymeric matrix, lysozyme (molecular weight, 14,000 Da; Sigma-Aldrich) as the antimicrobial active compound, glyoxal (40% Riedel de Haen, Gallarate, Italy) as the cross-linking agent, and glutaraldehyde (50%; Sigma-Aldrich) as the bonding agent.

A 13% (wt/vol) polyvinylalcohol-based autoclaved solution was cross-linked by adding 5 μl of glyoxal and hydrochloric acid (0.2 ml) as the reaction catalyst. The resulting solution, after homogenization at a moderate speed, was cast onto a Plexiglas plate. A solution of lysozyme (2%) dissolved in distilled water (10 ml) and the bonding agent was sprayed on the wet film to distribute the antimicrobial compound and the bonding agent uniformly. Glacial acetic acid (2 ml; Sigma-Aldrich) was the reaction catalyst and was also sprayed onto the film. The fabricated active film was dried under ambient conditions until the solvent was completely evaporated. The developed film had a thickness of ca. 100 μm. As a control, films of polyvinylalcohol containing glyoxal, glutaraldehyde, and glacial acetic acid without lysozyme were also prepared. Each developed active and control film was washed in 4.5 liters of distilled water to release the substances not bound to the film. Each of these films was then aseptically immersed in the respective test medium.

Bacterial strains. Five strains of *A. acidoterrestris*, isolated from spoiled fruit juice from the Laboratory of Applied Microbiology (Department of Food Science, University of Foggia) culture collection, were used. The cultures were grown on acidified malt extract agar (MEA, pH 4.5; Oxoid, Milan, Italy) at 44°C and then stored at 4°C as stock cultures for 1 month.

Culture media. To evaluate the antimicrobial effectiveness of the developed films, the following media were used: (i) acidified malt extract broth (MEB, pH 4.5; Oxoid) and (ii) commercial pasteurized apple juice (pH 3.5; sugar concentration, 11.1 °Brix).

Microbiological analysis. The microbiological analysis was conducted both on a single strain and on a five-strain cocktail of *A. acidoterrestris*. The culture medium in both cases was acidified MEB. Three volumes (150, 300, and 600 ml) of the selected medium were used.

For single-strain testing, *A. acidoterrestris* (PJ3) was grown at 44°C for 2 days on MEB. Dilutions of cell suspension were made in a sterile saline solution (0.9% NaCl) to obtain about 10^8 CFU/ml, and 150, 300, and 600 ml of acidified MEB were each inoculated with 1 ml of this suspension.

For testing with the five-strain cocktail, cells of each of the five strains of *A. acidoterrestris* were grown at 44°C for 2 days on MEB. Equal volumes of suspension of each strain were combined and diluted in sterile saline solution to obtain about 10^8 CFU/ml. Amounts of 150, 300, and 600 ml of the acidified MEB were each inoculated with 1 ml of the stirred culture cocktail mixture.

A control protocol that was identical in all respects to the above procedures was run, except that the film was not present in any of the tests.

In summary, microbiological tests for each medium volume were conducted on the following samples: (i) acidified MEB with active film, (ii) acidified MEB with lysozyme-free film, and (iii) acidified MEB without film.

All samples were incubated at 44°C and continuously stirred at a moderate speed with a stirrer (IKA KS 130 control, IKA Werke GmbH, Staufen, Germany). Periodically, a 0.1-ml aliquot was removed from each sample, serially diluted in a saline water solution, plated on acidified MEA, and incubated at 44°C for 48 h.

The preparation of *A. acidoterrestris* spore suspensions was performed according to Sinigaglia et al. *(19)*. Approximately 10^6 spores were inoculated into 500 ml of acidified MEB and 500 ml of commercial pasteurized apple juice. Trials with these media were run with active film, with lysozyme-free film, and without any film. Before adding the films to the respective medium, a 0.1-ml aliquot was removed from each sample and enumerated by dilution in a saline water solution on acidified MEA. After adding the films, a 0.1-ml aliquot was removed from each sample and periodically enumerated on MEA plates after 48 h of incubation.

Quantitative determination of film antimicrobial activity. The difference between the initial cell load and its value at 200 h (which was about the end of the period of observation) was taken as a quantitative measure of the antimicrobial efficacy of the investigated active compound. To calculate the Δ_{200} (the difference between the initial cell load and its value at 200 h of observation), a first-order kinetic equation that served as our model was fitted to the experimental data.

$$P(t) = P_\infty + (P_0 - P_\infty)\cdot\exp(-k\cdot t) \quad (1)$$

where $P(t)$ is the cell load at time t, P_∞ is the cell load at the stationary phase, P_0 is the initial value of the cell load, and k is the kinetic constant. Once the model's parameters were estimated, the antimicrobial efficacy could be calculated as follows:

$$\Delta_{200} = (P_0 - P_\infty)\cdot[1 - \exp(-k\cdot t)] \quad (2)$$

Even if it was possible to use equation 1 to estimate the confidence interval of each model's parameter, the confidence interval of Δ_{200} could not be estimated, because it does not correlate explicitly with the parameters found in equation 1. An alternative way to estimate the antimicrobial effectiveness of the investigated compound is to rearrange equation 1 in such a way that the Δ_{200} value appears directly as a parameter in the equation that models the kinetics of our experiment.

$$P(t) = P_0 + \Delta_{200}/[1 - \exp(-k\cdot 200)]\cdot[\exp(-k\cdot t) - 1] \quad (3)$$

By fitting equation 3 to the experimental data, it is possible to estimate the equation's parameters and their confidence intervals. Therefore, equation 3 was used in place of equation 1 to determine the antimicrobial activity of the developed active film and its confidence interval.

Statistical analysis. All analyses were carried out in duplicate by recording the results at two different times. Average values and standard deviations were calculated. The confidence intervals of the model's parameters were evaluated as follows: first, a fit was run with the original data; then, with the standard deviations of the data points, 100 additional fits were run on artificial data sets, which were generated by randomly varying the data around the fitted function. From these additional fits, a distribution of values for each parameter was determined. The sets of data ob-

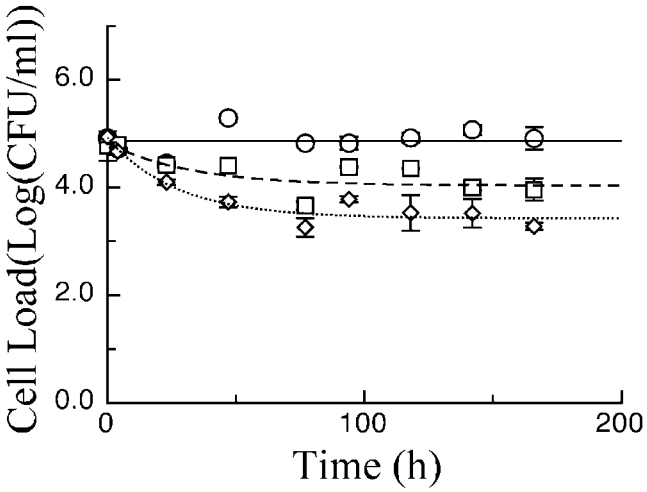

FIGURE 1. *Behavior of the single-strain* A. acidoterrestris *with respect to cell load in 600 ml of acidified MEB during storage.* ○*, Experimental data for acidified MEB without film;* ——*, fitting of equation 3 to the experimental data for tests without film;* □*, experimental data for acidified MEB with lysozyme-free film;* – – –*, fitting of equation 3 to the experimental data for tests with lysozyme-free film;* ◊*, experimental data for acidified MEB with active film;* ·····*fitting of equation 3 to the experimental data for tests with active film.*

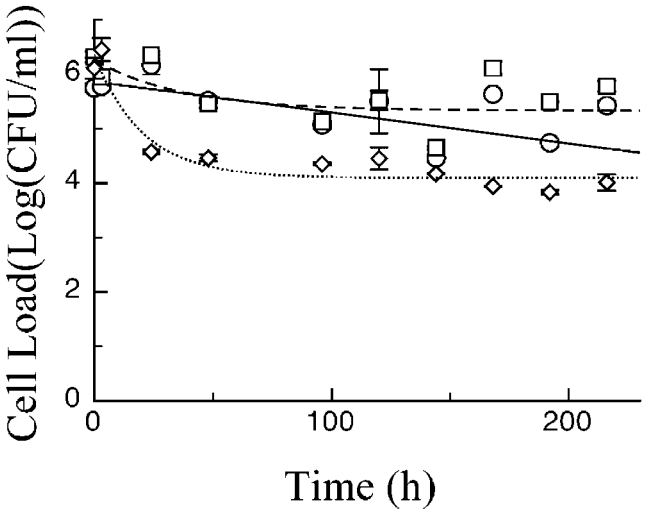

FIGURE 2. *Behavior of the five-strain* A. acidoterrestris *culture cocktail with respect to cell load in 600 ml of acidified MEB during storage.* ○*, Experimental data for acidified MEB without film;* ——*, fitting of equation 3 to the experimental data for tests without film;* □*, experimental data for acidified MEB with lysozyme-free film;* – – –*, fitting of equation 3 to the experimental data for tests with lysozyme-free film;* ◊*, experimental data for acidified MEB with active film;* ·····*fitting of equation 3 to the experimental data for tests with active film.*

tained for each parameter were statistically treated to determine the 95% confidence interval.

RESULTS AND DISCUSSION

The goal of this study was to assess the antimicrobial effectiveness of an active film against microorganisms that affect the quality of acidic beverages. The active compound was immobilized onto the film surface, and its antimicrobial activity against both a single strain and a five-strain cocktail of *A. acidoterrestris* was investigated. To investigate the influence of the medium on the antimicrobial activity of the developed films, two different media were used: (i) acidified MEB for both cells and spores and (ii) commercial apple juice only for spores. Moreover, to assess the influence of the ratio between the volume of cultural medium and the film surface on the performances of the active film, the volumes of the tested mediums ranged from 150 to 600 ml.

Figures 1 and 2 show the cell load plotted as a function of time for the single strain and the five-strain cocktail of *A. acidoterrestris,* respectively, in 600 ml of acidified MEB. There was a marked difference between the samples that contained the active film and the two controls; in particular, the curves for batch cultivations without film or with lysozyme-free films showed a slight decrease in the viable cell number. In contrast, for samples with the active films, a significant reduction in the viable cell concentration was observed immediately after inoculation; later, the cell load reached a stationary phase. To quantitatively determine the antimicrobial effectiveness of the active films, the Δ_{200} value was calculated. Two hundred hours was the maximum common period of observation for all investigated samples. As reported in ''Materials and Methods,'' a rearranged form of a first-order kinetic equation was used to fit the experimental data to estimate the equation's parameters and their confidence intervals. The values of Δ_{200} obtained from fitting the data to this equation, along with their respective confidence intervals for all investigated samples, are reported in Table 1. The values of Δ_{200} obtained for the active film were always higher than the values of Δ_{200} for the two corresponding control samples. Because the values of the confidence intervals for the parameters of all investigated samples did not superimpose each other, it is reasonable to assume that the differences among the investigated single strain and culture cocktail were statistically significant.

In Figure 3, the value of Δ_{200}, as a measure of the antimicrobial effectiveness of the active films, was plotted as a function of the ratio between the medium volume and the film area. As can be inferred from the data shown in the figure, in neither of the cases did the difference in the medium volume seem to influence the Δ_{200} parameter greatly. For this reason, the effectiveness of active film against *A. acidoterrestris* spores was evaluated with a single volume of medium, i.e., 500 ml. In Figure 4, the response of *A. acidoterrestris* spores with respect to cell load in acidified MEB during storage is shown. These data show that this microorganism germinated and grew in the samples with lysozyme-free film and without film; in contrast, there is a loss of viable spores in the presence of active films.

Because of the favorable results of the active compound in controlling *A. acidoterrestris* spores in acidified MEB, the same microbiological test was run on apple juice. The results, as shown in Figure 5, confirmed the inhibitory effect of the active film. Also, in these last two cases, a rearranged first-order kinetic equation was used to fit the data in order to estimate the values of Δ_{200} along with their respective confidence intervals. The fitting of data to equa-

TABLE 1. *Δ_{200} values obtained from the fitting of equation 3 to the experimental data, along with their respective confidence intervals (CI) for all investigated samples*

Alicyclobacillus acidoterrestris samples	Δ_{200} for cell load (95% CI)		
	Without film	With control film	With active film
Single strain in 150 ml of MEB	0.6870 (0.1055 to 1.2255)	1.3624 (0.9077 to 2.0720)	2.8459 (2.5458 to 3.2411)
Single strain in 300 ml of MEB	1.4155 (0.8820 to 1.8917)	0.5027 (5.3588×10^{-2} to 1.1140)	3.7938 (3.4967 to 4.1275)
Single strain in 600 ml of MEB	3.7863×10^{-16} (3.5956×10^{-16} to 3.8308×10^{-16})	0.7915 (0.5260 to 1.1561)	1.4859 (1.1951 to 1.7557)
Culture cocktail in 150 ml of MEB	0.9952 (0.6134 to 1.3687)	1.0973 (0.8907 to 1.4621)	2.5529 (2.1626 to 2.8291)
Culture cocktail in 300 ml of MEB	1.2688 (0.9671 to 1.6796)	1.1742 (0.8333 to 1.6123)	2.0161 (1.6905 to 2.3719)
Culture cocktail in 600 ml of MEB	1.1345 (0.8375 to 1.4015)	0.9306 (0.5890 to 1.2717)	2.2475 (1.8509 to 2.7281)
Single-strain spores in 500 ml of MEB	−1.0798 (−1.5741 to −0.8228)	−2.6380 (−3.1054 to −2.2384)	1.7121 (1.4860 to 2.0262)
Single-strain spores in 500 ml of juice	−1.6024 (−2.0262 to −1.3037)	−1.1381 (−1.4603 to −0.8543)	0.8067 (0.4225 to 1.1195)

tion 3 is shown in Figures 1 through 5, and the values of Δ_{200} are reported in Table 1. For the tests involving spores, the values of Δ_{200} for the two control samples were negative with respect to the corresponding active samples, indicating that *A. acidoterrestris* spores germinated and grew both in MEB and apple juice, whereas in the presence of active films, a significant loss of viable spores was observed.

Regarding the influence of the growth medium, the composition of the medium appeared to affect the efficacy of the active film in inhibiting microbial growth, as has also been reported in a previous study *(11)*, in which the effectiveness of a polyethylenoxide-like film containing Ag was tested against the same microorganism. In our study, when spores were inoculated into MEB, a rapid decrease in the viable spore count was observed until a stationary phase was attained. By contrast, in apple juice, a slow decrease in the number of viable spores was observed during the entire observation period, which was probably because the apple juice had a protective effect with respect to spore viability. According to the data reported in Table 1, the confidence intervals of the Δ_{200} parameters that were calculated for the two media were superimposed, suggesting that the differences in the investigated media are not statistically significant.

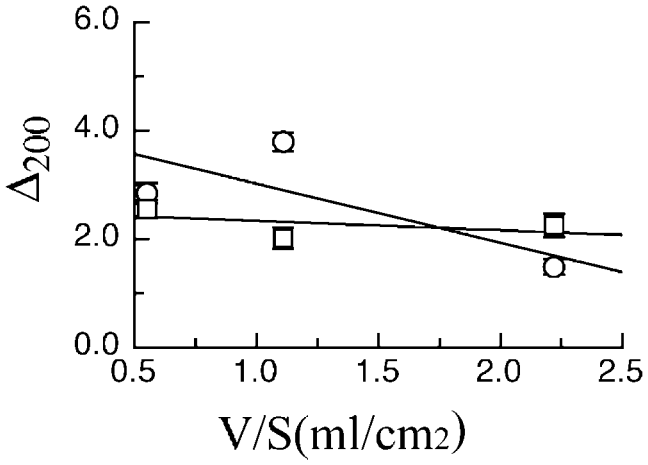

FIGURE 3. *Antimicrobial activity of the developed active films measured as the difference between the initial cell load and its value at 200 h (Δ_{200}) plotted as a function of the ratio between the medium volume and the film area.* ○, *Experimental data for active film immersed in inoculated medium by a single strain;* ——, *trend of the obtained experimental data;* □, *experimental data with active film immersed in inoculated medium by a culture cocktail;* ——, *trend of the obtained experimental data.*

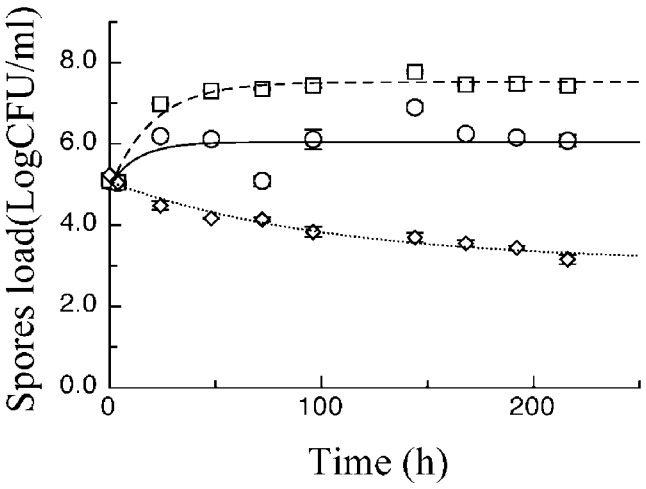

FIGURE 4. *Behavior of* A. acidoterrestris *spores with respect to cell load in acidified MEB during storage.* ○, *Experimental data for acidified MEB without film;* ——, *fitting of equation 3 to the experimental data for tests without film;* □, *experimental data for acidified MEB with lysozyme-free film;* – – –, *fitting of equation 3 to the experimental data for tests with lysozyme-free film;* ◇, *experimental data for acidified MEB with active film;* ·····*fitting of equation 3 to the experimental data for tests with active film.*

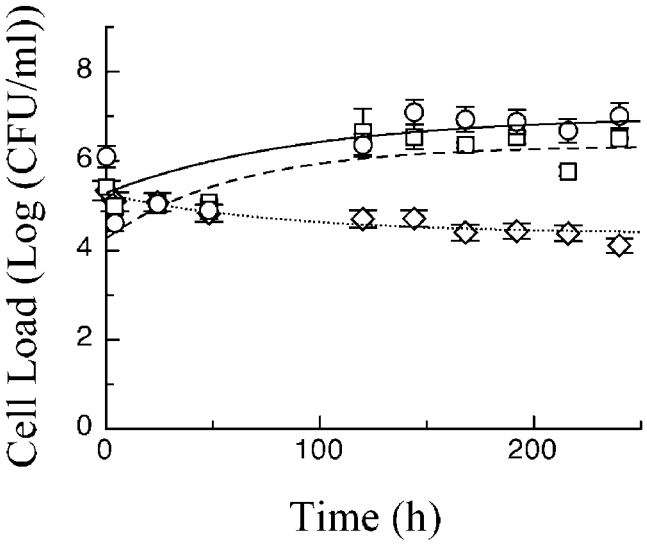

FIGURE 5. *Behavior of* A. acidoterrestris *spores with respect to cell load in commercial apple juice during storage.* ○, *Experimental data for juice without film;* ——, *fitting of equation 3 to the experimental data for tests without film;* □, *experimental data for juice with lysozyme-free film;* – – –, *fitting of equation 3 to the experimental data for tests with lysozyme-free film;* ◇, *experimental data for juice with active film;* ·····*fitting of equation 3 to the experimental data for tests with active film.*

In conclusion, our results suggest that the proposed active film can be successfully used to inhibit the growth of viable vegetative cells of *A. acidoterrestris* and maintain its efficacy for various medium volumes. The antimicrobial effectiveness of the developed active system, tested also on the spores, confirmed the ability of the immobilized lysozyme to inhibit the spores of this microorganism. The results of this study suggest the possibility of applying this packaging system to acidified fruit juices because of its ability to inhibit the germination of the spores of this potent spoilage microorganism even better than its vegetative cells.

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